The Power of Replicates

Introduction

Carefully designing and controlling experiments is as important as the execution of the experiment itself. One approach that ensures greater experimental success in gene expression studies using microarrays is the incorporation of replicates. Replication of conditions lends statistical power that increases the confidence of the conclusions drawn from these experiments. This text discusses the many ways in which researchers can benefit from using replicates in their studies.

In a typical gene expression study, researchers are interested in genes expressed above background levels, and genes that are differentially expressed between conditions of interest. The variation present in microarray data poses the challenge of determining whether differences between expression measurements are caused by biological differences, or by statistical chance. The best way to address this challenge is to use replicates for each condition studied. There are two primary types of replicates: technical and biological. Technical replicates involve taking one sample from the same source tube, and analyzing it across multiple conditions, e.g., analyzing one sample six times across multiple arrays. Biological replicates are different samples measured across multiple conditions, e.g., six different human samples across six arrays.

Using replicates offers three major advantages:

- **Replicates can be used to measure variation in the experiment so that statistical tests can be applied to evaluate differences.**
- **Averaging across replicates increases the precision of gene expression measurements and allows smaller changes to be detected.**
- **Replicates can be compared to locate outlier results that may occur due to aberrations within the array, the sample, or the experimental procedure.**

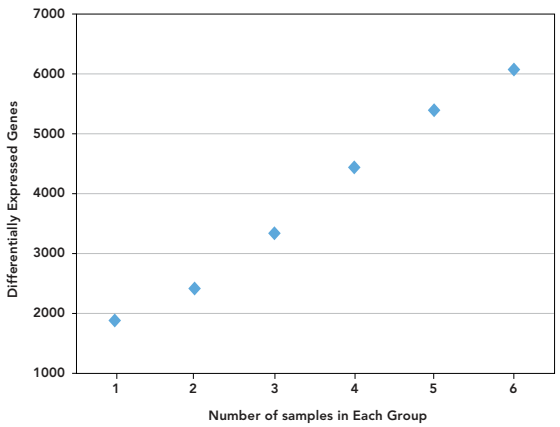
The high cost of microarrays has typically constrained or eliminated the number of replicates in most studies. However, the cost must be evaluated against the quality of the data, which includes ease of use, initial financial outlay, cost of arrays and reagents, and experimental design (e.g., replicates assayed). A more informative experiment may be achieved by assaying a smaller set of test conditions while including more replicates rather than assaying a larger set of test conditions with fewer replicates.

BENEFITS

A. Measure Variation

Replicates improve the measurement of variation. Normally, if only one array exists per condition, then fold change is used to determine differential expression. However, the variation of the expression level for each gene is different and unknown. Multiple studies have shown that fold change on its own is an unreliable indicator[1,2]. If multiple measurements (i.e., replicates) exist for each gene within each condition, the measurement of variation can be estimated. If the data follow an approximately normal distribution, the t-test or its variants reveal significant differential expression. If the data distribution is unclear, non-parametric tests such as the Mann-Whitney test can be applied. Several publications make specific recommendations on the number of replicates required to detect various fold changes[3,4].

Figure 1: Differentially Expressed Genes As A Function Of Replicates



Increasing the number of biological replicates in each group enhances the power to detect differentially expressed genes.

B. Increase Precision

Averaging across replicates enhances the precision of measurements. If the standard deviation of an expression measurement is s, then the standard deviation of the average across n replicates is. As the number of replicates increases, both the detectable difference from background and the detectable fold change decrease.

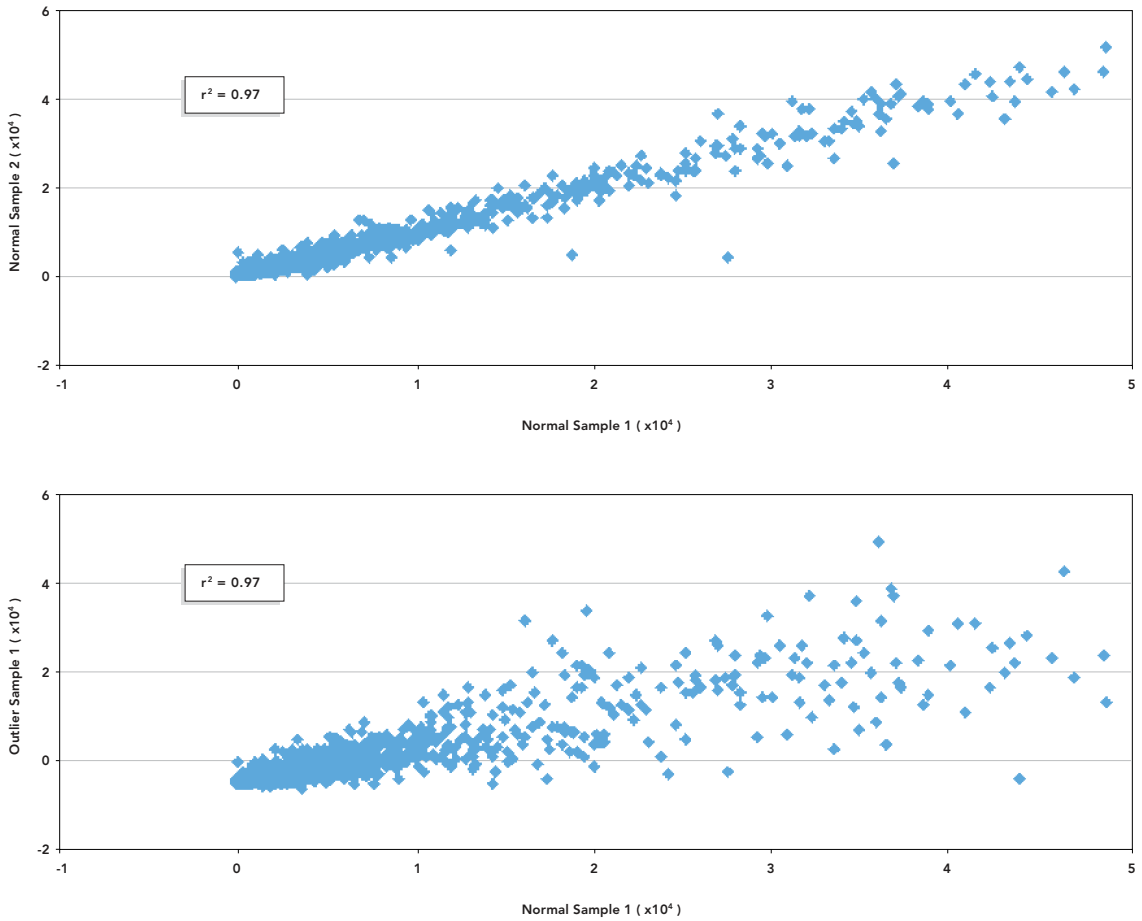
C. Detect Outliers

The presence of outlier samples can have a severe impact on the interpretation of data. Most array platforms have internal controls that detect various problems in an experiment. However, internal controls may not identify all issues. A more powerful approach is also to consider the correlation between replicates. Subtle problems with the array, the sample, or the experimental procedure often become obvious in a pair-wise plot of replicate measurements.

Data

To illustrate the points above, a data set of 12 samples were analyzed on the Illumina Human Whole-Genome Expression BeadChips. The samples included six biological replicates from normal tissue and six biological replicates from diseased tissue. Figure 1 illustrates the number of differentially expressed genes as a function of the number of samples in each group. When the number of samples was two or more, a standard t-test was applied with a false positive rate of 0.05. For one sample in each group, fold change was used to determine dif-

Figure 2: Identification Of Outliers



By plotting correlation plots between replicates, outlier samples may be identified (top) that are not apparent without a replicate comparison (bottom).

Table 1: Differently Expressed Genes Identified Under Various Conditions

	Between Normal Samples	Between Normal And Disease
Fold change, no outlier	554	1,906
t-test: 3 replicates, no outlier	559	3,377
Fold change with outlier	1,898	3,259
t-test: 3 replicates with outlier	503	2,640

ferential expression. The fold change required for significance was set to achieve the same false positive rate as that used in the t-test.

The effect and treatment of outliers on a data set can be illustrated as follows. Normal samples were divided into two groups of three. Three disease samples were selected. Random noise was added to one of the normal samples to simulate an outlier, and differential expression was assessed between the two groups of normal samples and between the normal and disease samples. Differential expression was determined via fold change and then by t-test employing replicates. Table 1 shows the number of differentially expressed genes identified under the various conditions.

Genes differentially expressed between normal samples were false positives. In the fold-change analysis with no replicates, the false positive rate increases dramatically if the sample analyzed was an outlier.

Using the t-test, we can estimate the noise in the data, so that less differential expression was detected between conditions, but the false positive rate remained the same. Figure 2 shows that correlation plots between replicates would have revealed the outlier sample.

Conclusion

With the increased density of current microarrays, gene expression researchers must be able to discern between true differences across experimental and controlled samples, and those caused by random variation. As the above data demonstrate, an experimental design that employs replicates allows researchers to detect variation within the assay, increases the precision of measurement when comparing data points, and provides the ability to detect misleading and irrelevant outliers.

References

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